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Effects of High-Temperature Stress on Various Biomembranes of Leaf Cells *In Situ* and *In Vitro*

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ABSTRACT

The sensitivity of photosynthetic and respiratory functions to supraoptimal temperature stress was compared after heating of leaves, protoplasts and membrane systems of spinach (*Spinacia oleracea* L. cv. Monatol) and lettuce (*Valerianella locusta* [L.] Betcke) *in situ* and *in vitro*.

After heating of whole leaves or protoplasts, endogenous respiration was not or only slightly affected at temperatures which caused a marked decrease of photosynthesis. This was manifested when mitochondria and thylakoids were isolated from heat-treated leaves. In the presence of exogenous substrates, mitochondrial electron transport and phosphorylation were even somewhat stimulated compared to the controls.

Inactivation of net CO_2 uptake of whole leaves following heat stress and of the photochemical activities of chloroplast membranes isolated from heat-treated leaves of the same origin occurred nearly simultaneously. In protoplasts, photosynthesis was inactivated at temperatures far below those which caused drastic changes in the integrity of the tonoplast and the plasmalemma. This indicates that damage occurring within the chloroplasts rather than alterations in the compartmentation of the cell is responsible for the high sensitivity of photosynthesis to supraoptimal temperature stress.

Mitochondria and thykaloids isolated from the same preparation of intact leaves under comparable conditions and subjected to heat treatment *in vitro*, however, were inactivated nearly in the same temperature range. Thus, mitochondria are much more stable within their cytoplasmic environment.

Cellular membranes are thought to be primarily involved in injury caused in plant cells by extreme temperatures. The question arises as to how various membrane systems differ in thermolability. In green tissues, respiration is more heat-stable than photosynthesis (1, 3, 14, 15, 17). Investigations with isolated membrane systems have shown that heat treatment of chloroplasts results primarily in an inactivation of the photochemical reactions in the highly thermolabile thylakoid membranes (4, 23, 24). On the other hand, isolated plant mitochondria also became inactivated even by mild heat treatment (5, 13, 25). Comparison of these studies is difficult as measurements of the thermal stability of chloroplasts and mitochondria have been performed with different plant material and under variant conditions. Moreover, there are hints that other cellular membranes such as the plasmalemma also are susceptible to high temperature stress (10, 19). Thus, heat damage to energy-conserving processes *in situ* may result from a heat-induced loss of cellular compartmentation.

The effect of supraoptimal temperatures on respiratory and photosynthetic functions of spinach leaves has been investigated more thoroughly in the present study. The overall processes of net photosynthesis and respiration of whole leaves were compared after they had been subjected to various temperatures. To study alterations in cellular membranes after heating *in situ*, intact leaves were exposed to various elevated temperatures; subsequently, mitochondria and thylakoids were isolated and their respiratory and photosynthetic activities were determined. Isolated protoplasts from lettuce leaves have been used to compare the integrity of tonoplast and plasmalemma with the activity of photosynthesis and respiration after a high temperature treatment. For investigation of the effect of heat on membrane systems *in vitro*, mitochondria and thylakoids were isolated from one leaf batch in similar isolation media. After supraoptimal temperature treatment under identical conditions, the biochemical activities of the membrane systems were measured.

MATERIALS AND METHODS

Plant Material. Spinach leaves (*Spinacia oleracea* L. cv. Monatol) were harvested from 4 to 6-week-old plants grown in soil culture in a greenhouse at temperatures around 20°C and a photoperiod of usually 10 h light/14 h dark. Lettuce leaves (*Valerianella locusta* [L.] Betcke) were collected during March to May from 4-week-old plants grown under conditions similar to those used for spinach but kept under natural photoperiod.

Isolation of Mitochondria and Thylakoids. Both biomembrane systems were isolated from intact and heat-treated spinach leaves in a single procedure as described earlier (26). The washing medium always contained mannitol (300 mM) as osmotic agent. After isolation, mitochondria and thylakoids were resuspended and stored at 0°C in a medium consisting of 300 mM mannitol, 15 mM Hepes-NaOH, 15 mM 2-*N*-morpholino-propane sulfonic acid-NaOH, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM EDTA, and 0.4% BSA (pH 7.2).

Isolation of Protoplasts. Protoplasts were isolated from intact spinach and lettuce leaves by a method developed by Edwards *et al.* (7) and modified by Hartung *et al.* (11) except for the composition of the digestion medium which contained 500 mM sorbitol, 1 mM $CaCl_2$, 5 mM Mes-KOH (pH 5.5), 0.5% PVP (mol wt 25,000), 1% BSA, 0.5% Cellulase Onozuka R-10, and 0.5% mazerozym R-10 (both from Welding and Co., Hamburg). The protoplasts were stored in the dark at 0°C in a medium containing 500 mM sorbitol, 1 mM $CaCl_2$, 5 mM Mes-KOH (pH 5.5), 0.5% PVP (mol wt 25,000), 1% BSA, 0.5% Cellulase Onozuka R-10, and 0.5% macerozym R-10 (both from Welding and Co., Hamburg). The protoplasts were to Arnon (2). The protein content of the mitochondrial fraction was evaluated as described by Lowry *et al.* (18); correction for contamination with thylakoid protein was carried out as proposed by Douce *et al.* (6).

Heat Treatment of Intact Leaves. Detached leaves were incubated for 6 min in a water bath at temperatures ranging from 36 to 43°C. Prior to CO_2 gas exchange measurements, leaves were allowed to recover for at least 90 min at room temperature (20–24°C) at which the controls were kept during the whole time (see Ref. 29). When mitochondria and thylakoids were isolated after

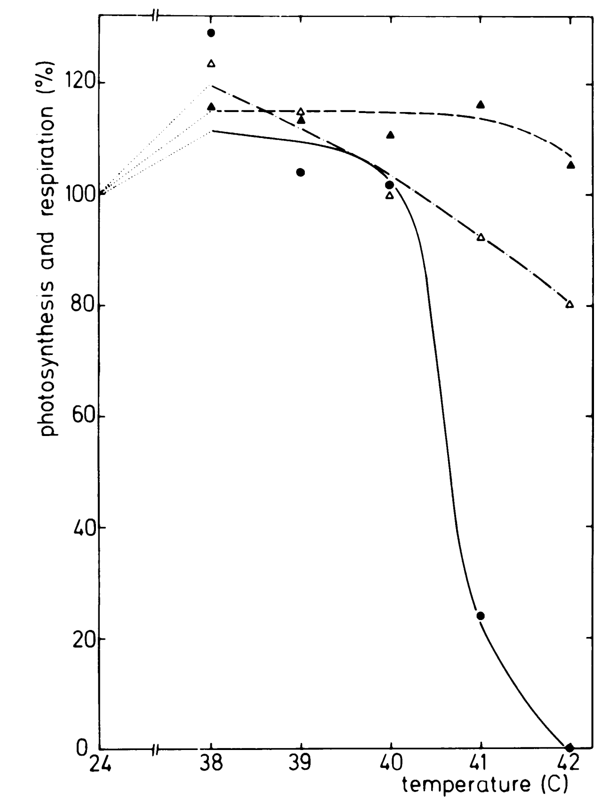

FIG. 1. Photosynthesis and respiration of spinach leaves after incubation for 6 min at temperatures indicated on the abscissa. After heat treatment, the leaves were stored at 24°C for at least 90 min either in the dark before measuring of respiration or in artificial room light and special preillumination (60-w incandescent lamp through a 10-cm water layer) for 20 to 30 min before measuring of net photosynthesis and, subsequently, respiration. Ordinate: activities in percentage of the controls kept at 24°C. Activities of the controls (=100%) in μmol CO_2 mg^{-1} Chl h^{-1}: net photosynthesis (●), 72.3; respiration after photosynthesis (△), 24.2; respiration after a dark period (▲), 18.9.

high temperature treatment of the plant material, the heated leaves were first kept for about 15 min at room temperature and then prechilled for 10 min at 4°C before isolation was started. During the recovery periods, leaf-stalks were kept in water.

Heat Treatment of Protoplasts. Aliquots of 70 to 100 μl of the protoplast suspension (corresponding to 25–40 μg Chl) were added to 0.7 to 0.8 ml of the protoplast reaction medium (see below) which was already brought to 37 to 56°C, respectively. After incubation for 3 min at the respective temperatures, the suspension was transferred for 2 to 5 min in a water bath at 20°C. Subsequently, photosynthetic and respiratory activities were measured as outlined below. Controls were kept at 20°C.

Temperature Treatment of Membrane Systems Isolated from Intact Leaves. Small samples (0.3 ml each) of the mitochondria and thylakoid suspensions were incubated for 3 min in a water bath at temperatures ranging from 30 to 45°C and then transferred for 2 min to 20°C. Controls were kept at 20°C during that time.

Photosynthesis and Respiration of Whole Leaves. CO_2 gas exchange was measured with a gas analyzer (Maihak UNOR-SN 1) as described by Weis (29). The construction of the lucite chamber allowed a continuous gas stream to pass both sides of the leaf area. Measurements took place at room temperature (23–24°C) in air containing 300 μl CO_2/L at a flow rate of 20 L/h. Illumination was performed with red light provided by a 240-w halogen lamp and filtered through a 2-cm water layer, a RG 630 cutoff filter (Schott and Gen., Mainz), an IR-absorbing filter Calflex C (Balzers, Liechtenstein), and an 8-mm heat absorption filter KG 3 (Schott and Gen., Mainz). The light intensity was 225 w/m^2.

Photosynthetic and Respiratory Activities of Isolated Protoplasts, Mitochondria, and Thylakoids. Measurements were performed polarographically with a Clark-type O_2 electrode at 20°C. The reaction medium for protoplasts contained 500 mM sorbitol, 1 mM $CaCl_2$, and 30 mM Hepes-KOH (pH 7.6). Their endogenous respiration was measured in the dark before and after photosynthesis took place. The addition of various exogenous substrates did not stimulate O_2 uptake in the dark probably due to permeability barriers of the plasmalemma. Photosynthesis was started by addition of 2 mM $NaHCO_3$ and illumination with saturating red light (550 w/m^2) provided by a 150-w halogen lamp using a filter combination as described earlier (26). The presence of 1 mM $CaCl_2$

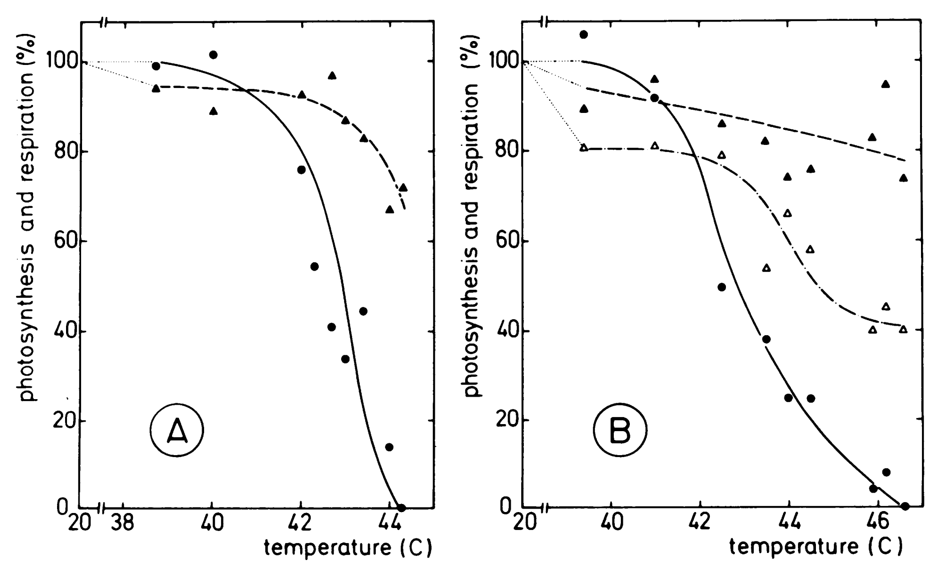

FIG. 2. Photosynthesis and respiration of spinach (A) and lettuce (B) leaf protoplasts after 3 min heating at temperatures indicated on the abscissae. Ordinates: activities in percentage of the controls kept at 20°C. Rates of the controls (=100%) in μmol O_2 mg^{-1} Chl h^{-1}: net photosynthesis (●), 69.2 (A) and 97.3 (B); respiration before photosynthesis (▲), 11.8 (A) and 8.0 (B); respiration after photosynthesis (△), 18.0 (B).

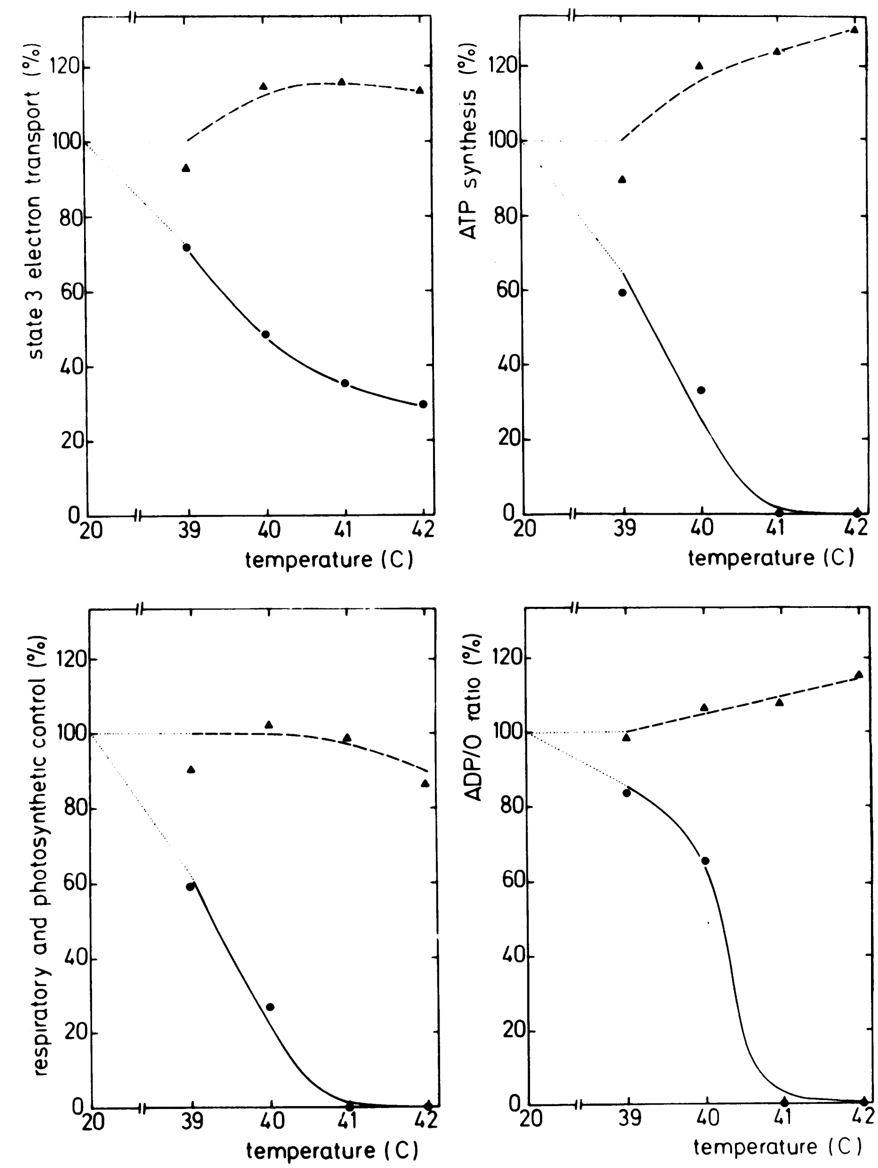

FIG. 3. Respiratory and photosynthetic activities of mitochondria (▲) and thylakoids (●) isolated from spinach leaves which were subjected prior to the isolation procedure to a 6 min heat treatment at temperatures indicated on the abscissae. Ordinates: activities in percent of the controls which were isolated from leaves kept at 20°C. Activities of the controls (=100%): Mitochondria: 26 (72) nmol O_2 (ATP) mg^{-1} protein min^{-1}; RC, 2.61; ADP:O ratio, 1.40. Thylakoids: 70 (129) μmol O_2 (ATP) mg^{-1} Chl h^{-1}; photosynthetic control, 3.14; ADP:O ratio, 0.94.

completely inhibited the HCO_3^--dependent O_2 evolution of free intact chloroplasts contaminating the preparation. The O_2 consumption of mitochondria was measured as outlined by Douce *et al.* (6) using 40 mM malate plus 25 mM glutamate as substrate. Methylviologen-mediated photosynthetic electron transport of thylakoids was determined in the presence of KCN under conditions described earlier (26). ATP synthesis, respiratory control, photosynthetic control, and ADP:O ratios were calculated according to Estabrook (8) and Robinson and Wiskich (20).

Assay for Tonoplast and Plasmalemma Alterations in Isolated Protoplasts. The breakdown in the permeability of the plasmalemma was observed by staining the protoplasts with Evan's blue (9, 21). Tonoplast alteration was evaluated by the loss of the ability of the vacuoles to accumulate neutral red (16). In both cases, protoplasts were suspended after heat exposure at room temperature in the same reaction medium (pH 7.6) used during heat treatment with either Evans blue or neutral red added to final concentrations of 0.5 or 0.05%, respectively. Counting of the percentage of stained protoplasts was performed in a Thoma chamber under a brightfield microscope.

RESULTS

At temperatures which caused an inactivation of net photosynthesis of intact leaves, respiration was not appreciably impaired (Fig. 1). However, there are small differences in respiration activity found after storage of heat-treated leaves either in the dark or in the light. These might be due to differences in the availability of substrates in the cells which may be abolished after heat-induced inactivation of photosynthesis. The absolute rates of CO_2 release of the respective controls favor this conclusion: illumination with photosynthetically active light preceding respiration measurements may cause a better supply of mitochondria by carbohydrate intermediates.

The result obtained with intact leaves was confirmed with isolated leaf protoplasts (Fig. 2). In both spinach and lettuce protoplasts, heating for 3 min at temperatures up to 44 to 47°C

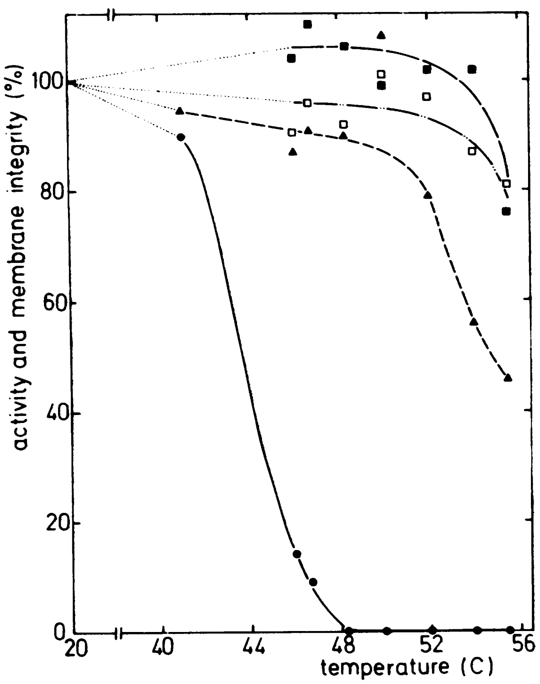

FIG. 4. Photosynthesis, respiration, and integrity of tonoplast and plasmalemma of lettuce leaf protoplasts after 3 min heating at temperatures indicated on the abscissa. Ordinate: activities and membrane integrities in percent of the controls kept at 20°C. Control values (=100%): net photosynthesis (●), 90.4 μmol O_2 release mg^{-1} Chl h^{-1}; respiration before photosynthesis (▲), 5.5 μmol O_2 uptake mg^{-1} Chl h^{-1}; tonoplast integrity (□), 91%; plasmalemma integrity (■), 78%.

caused a complete breakdown of photosynthesis while endogenous respiration measured after a dark period still maintained 70% to 80% of the rates of the controls. The more pronounced decrease in O_2 uptake determined after photosynthesis (Fig. 2B) can probably be explained similarly as outlined for whole spinach leaves (Fig. 1). Inasmuch as both intact leaves and protoplasts were equally affected by heat stress, changes in stomatal conductance may not be responsible for the decrease in CO_2 uptake in the light.

The marked thermolability of photosynthesis was apparent if mitochondria and chloroplast membranes were isolated after an *in situ* heat treatment of spinach leaves (Fig. 3). At temperatures at which photophosphorylation was already completely inactivated, neither RC nor ADP:O values of mitochondria decreased but were sometimes even slightly stimulated compared to the controls. This indicates that energetic efficiency of respiration was not impaired. Thus, thermal inactivation of leaf cells is due to damage of the chloroplast membranes rather than to alterations in the mitochondria.

The question arises whether heat inactivation of chloroplast membranes and, thus, photosynthesis is correlated with alterations in the integrity of plasmamembranes, *e.g.* with a breakdown of cellular compartmentation. To test this, heat-treated protoplasts were incubated at room temperature in solutions containing either neutral red or Evans blue which can be used as specific indicators for the intactness of the tonoplast and the plasmalemma, respectively. Living protoplasts exclude Evans blue; staining of the cells occurs only when the plasmalemma has been damaged (9, 21). Neutral red enters the vacuole of intact protoplasts in the uncharged form and is protonated due to the lower vacuolar pH; the bright red colored ionic species cannot pass the tonoplast and is, therefore, accumulated in the vacuole (16).

Figure 4 demonstrates that photosynthesis and respiration already became markedly inactivated before drastic changes in the integrity of tonoplast and plasmalemma occurred. At a temperature which completely inactivates net photosynthesis, the protoplasts excluded Evans blue and accumulated neutral red to the same extent as the unheated controls. When heat treatment of protoplasts was more pronounced (3 min at 55.5°C) and even respiration decreased to about 50%, only slight changes in tonoplast and plasmalemma intactness have been observed. Only after exposure of protoplasts to extremely high temperatures, *e.g.* 3 min at 70°C, all protoplasts were either stained with Evans blue or none of the vacuoles showed any accumulation of neutral red indicating a complete damage of the plasma membranes. Thus, comparison in the relative thermostability of cellular membranes in intact leaves and protoplasts showed that thylakoids are considerably more heat-sensitive than mitochondria and plasma membranes.

Since it was shown in numerous papers that isolated thylakoids and mitochondria are very sensitive to high temperature treatment, attempts have been made to compare the thermostability of these membrane systems *in vitro*. For this, it was necessary to isolate chloroplasts and mitochondria under comparable conditions, *i.e.* from the same leaf material in a single isolation procedure. Small differences in the composition of the isolation and washing media were allowed in order to maintain functional integrity of the membrane systems. Storage and heat treatment, however, were always performed in a medium which had the same composition for both thylakoids and mitochondria.

The effect of heat on isolated membrane systems is in part not in agreement with the results obtained after high temperature treatment *in situ*. Photosynthetic and respiratory functions of the membranes became inactivated in the same temperature range and showed nearly the same pattern of inactivation (Fig. 5). In both mitochondria and thylakoids, ATP synthesis and electron transport decreased with increasing temperature. Phosphorylation was more sensitive than electron transport; this can be seen also from the reduced ADP:O ratios. The decrease in RC and photosynthetic control values with increasing heat stress was due to an inhibition of electron transport in state 3 rather than to a relative increase in state 4. Energetic coupling of both isolated membrane systems was almost completely inactivated at temperatures between 40 and 45°C.

DISCUSSION

The data presented in this paper clearly show that after exposure of both whole leaves (Fig. 1) and isolated protoplasts (Figs. 2 and 4) to elevated temperatures photosynthesis is much more impaired than respiration. To examine whether this different heat sensitivity of the overall processes is manifested in the energetic coupling of chloroplasts and mitochondria, isolated membrane systems have been investigated. When whole leaves were subjected to heat stress leading to an incipient inactivation of the energy-conserving reactions within the chloroplasts, the respiratory activities of mitochondria were unimpaired or even stimulated (Fig. 3). This may reflect a high-temperature-induced alteration of the physiological state of the inner mitochondrial membrane in the course of heat treatment *in situ* which persisted even during subsequent isolation of the organelles at temperatures close to the freezing point. Why heating in their cytoplasmic surroundings affects mitochondria and thylakoids differently is not clear.

Data obtained after heat treatment of isolated leaf protoplasts indicate that drastic changes in the permeability of the tonoplast and the plasmalemma take place only at temperatures far above those which cause an inactivation of photosynthesis (Fig. 4). This is in agreement with earlier findings based upon the solute leakage technique (4, 17) and favors the conclusion that heat inactivation of photosynthesis occurs at the chloroplast level rather than by changes in the permeability of plasma membranes or even a breakdown of cellular compartmentation. Nevertheless, it is possible that leakage of smaller molecules or ions, *e.g.* such as K^+

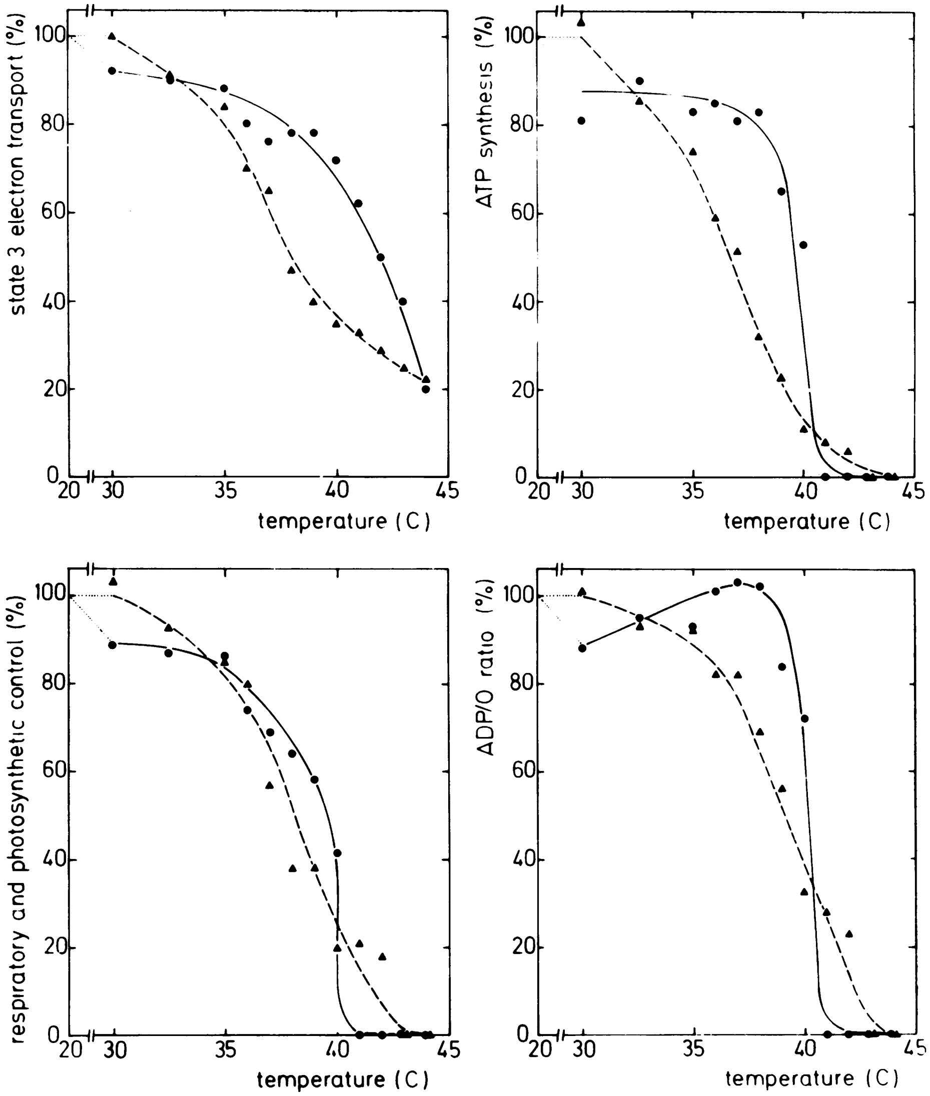

FIG. 5. Respiratory and photosynthetic activities of mitochondria (▲) and thylakoids (●) isolated from spinach leaves and exposed for 3 min *in vitro* to temperatures indicated on the abscissae. Ordinates: activities in percent of the controls kept at 20°C. Activities of the controls (=100%): Mitochondria: 25 (72) nmol O_2 (ATP) mg^{-1} protein min^{-1}; RC, 2.22, ADP:O ratio, 1.42. Thylakoids: 50 (89) μmol O_2 (ATP) mg^{-1} Chl h^{-1}; photosynthetic control, 2.11; ADP:O ratio, 0.92.

(see Ref. 10), may occur at temperatures lower than those causing the plasmalemma to become permeable for the large molecule of Evans blue (mol wt 960). Thus, thylakoids are among the most heat-sensitive cellular membrane systems in green plant cells.

Although the absolute temperature at which photosynthesis is inactivated depends on the respective material, heating of leaves from the same origin resulted in a nearly simultaneous breakdown of net CO_2 uptake of the whole leaves (Fig. 1) and of photosynthetic activities of thylakoids isolated after an *in situ* heat treatment (Fig. 3). Moreover, a marked decrease of photosynthetic activities has been observed at a certain temperature range independently of whether heat treatment took place *in situ* (Fig. 3) or *in vitro* (Fig. 5). Under both conditions, energy-conserving reactions of isolated thylakoids show the same pattern of inactivation. The finding that thylakoid membranes are highly thermolabile is in accordance with earlier results (4, 23, 24). Whereas heat sensitivity of the thylakoid membrane is well established, most soluble enzymes of the Calvin cycle are relatively stable towards high temperatures (4, 23). However, some light-activated stroma enzymes become inhibited in the same temperature range as the overall process of photosynthesis, possibly due to a heat inhibition of PSII (4). Recently, Weis (29, 30) has shown that after mild heat treatment, the inactivation of CO_2 fixation which is due to an inhibition of light-activation of ribulose-1,5-bisphosphate carboxylase was completely reversible during subsequent storage of the leaves at room temperature. Therefore, it is still unclear whether heat inactivation of photosynthesis is primarily due to damage of thylakoid membranes or of soluble enzymes within the chloroplasts.

In contrast to the results obtained with chloroplast membranes, mitochondria proved to be substantially less stable when exposure to high temperatures took place *in vitro* than *in situ*, *i.e.* in their natural cytoplasmic environment (Figs. 3 and 5). This may have several reasons. Similar to the results obtained with isolated thylakoids (27), certain membrane components may be released from the inner mitochondrial membrane during heating *in vitro*, whereas in the cell they might be protected by surrounding proteins of the intramembrane space or by cytoplasmic constituents. Moreover, as was shown for thylakoids (12, 22), the extent of mitochondrial inactivation may depend on the pH and the nature and concentration of the compounds present in the medium in which heat treatment takes place. Since plant mitochondrial malate dehydrogenase (28) and other soluble enzymes (23) exhibit high thermal stability, heat inactivation may result from alterations within the complex membrane-bound electron transport chain itself. This is in accordance with earlier results concerning the effect of heat treatment on isolated plant mitochondria from different origin (5).

From the data discussed above we concluded that heat injury of leaves is primarily due to irreversible alterations in the photosynthetic apparatus. It is likely that thylakoids are among the most heat-sensitive sites within the chloroplasts. Although it was shown that experiments using isolated organelles can be variable and not representative of the *in vivo* treatment effects, isolated thylakoids turned out to be suitable for investigation of the primary mechanism of heat inactivation to chloroplast membranes *in situ*.

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